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Several protein kinases involved in mammary gland development have been associated with human breast cancer and murine animal models of mammary gland carcinogenesis. To identify other protein kinases expressed in the mammary gland which may be involved in these processes, an RT-PCR degenerate oligonucleotide screen was performed on several transformed mammary epithelial cell lines and tissue derived from the mammary gland during different developmental stages. Forty-one kinases were identified, of which three were novel. One of these novel kinases, Krct, does not belong to any previously described subfamily of kinases. Analysis of the expression of murine Krct, demonstrates Krct expression in all tissues analyzed during all stages of development. Despite this wide expression pattern, Krct expression is preferentially expressed in the epithelium of several tissues including the mammary gland. Since many kinases regulate cellular proliferation and differentiation, it will be important to determine Krct's role in these processes. An analysis of the expression pattern of Krct during proliferation and differentiation has been initiated. Preliminary results demonstrate Krct may be cell cycle regulated. Since many genes that regulate the cell cycle are themselves regulated, these results suggest that Krct may regulate proliferation. Further analysis of Krct with proliferation and differentiation assays *in vitro* and *in vivo* will determine if Krct is involved in these cellular processes.

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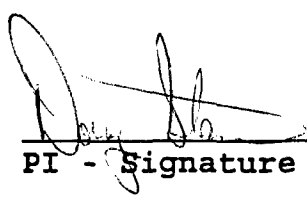

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Introduction:

Epidemiological evidence suggests breast cancer risk is closely related to development of the mammary gland. Additionally, development of the mammary gland through fetal life, puberty, pregnancy and post-lactational regression involves cellular processes such as differentiation, proliferation and apoptosis(1-3). Protein kinases are key regulatory molecules of these cellular processes(4). Several protein kinases have been shown to contribute to mammary gland development and breast cancer, including c-erb-B2/Neu, the epidermal growth factor (EGF) receptor, c-Src and Met(4-11).

To identify other kinases involved in both of these processes, we performed RT-PCR using degenerate oligonucleotides to catalytic subdomains VII and IX conserved in all protein kinases. Forty-one kinases were identified after screening several transformed mammary epithelial cell lines and developmental time points in the mammary gland by this method. Three of these kinases were novel, one of which, H51, does not fit into any known kinase subfamily and has closest homology to a serine/threonine kinase in yeast with unknown function.

The H51 PCR product obtained from this screen was used to isolate a full-length cDNA. The kinase encoded by this cDNA was renamed from H51 to Krct and will henceforth be referred to as such. After isolation of full length *Krct*, analysis of its expression pattern, chromosomal localization and verification of its phosphotransferase activity were performed and published(12). Since many kinases are involved in cellular proliferation and differentiation, we hypothesize that Krct may also be involved in these processes. To investigate this hypothesis, *in vitro* and *in vivo* proliferation and differentiation assays will be performed.

Specific Aims:

Aim 1: Determine the role played by Krct in proliferation.

- **A. Generate antisera specific for Krct.**
- **B. Characterize the expression of Krct *in vitro* as a function of cell proliferation.**
- **C. Overexpress Krct to assess its effects on proliferation.**
  - **i. Assessing the effects of Krct overexpression on proliferation *in vitro*.**
  - **ii. Assessing the effects of Krct overexpression on proliferation *in vivo*.**

Aim 2: Determine the role played by Krct in differentiation.

- **A. Characterize the expression of Krct in an *in vitro* differentiation system.**
- ***B.* Overexpress the novel kinase, Krct, to assess its effects on differentiation *in vitro*.**
- **C. Overexpress the novel kinase, Krct, to assess its effects on differentiation *in vivo*.**

Aim 3: Determine the potential role played by Krct in carcinogenesis in the mammary gland.

- **A. Assessing the effects of Krct on transformation *in vitro*.**
- **B. Assessing the tumorigenicity of Krct overexpressing cells.**
- **C. Determine the effects of Krct overexpression on carcinogenesis *in vivo*.**

Task1. Determine the role played by Krct in proliferation: months 1-36.

- Generate antisera to Krct: months 1-14.
- Generate pTetO.Krct and pTetO.LacZ constructs: months 1-7.
- Characterize Krct expression during proliferation in HC11 cell: months 8-16.
- Generate and characterize stable transfectants in HC11 cells: months 17-24.
- Perform Northern and Western analysis to assess proliferation rates: months 25-36.
- Perform FACS and 3H-Thymidine studies to assess proliferation rates: months 25-36.
- Create MMTV.Krct transgenic construct: months 1-6.
- Create founder mice for transgenic construct: months 7-10.
- Characterize changes in proliferation rates in transgenic mice by *in situ* hybridization, whole mount analysis, and hematoxylin and eosin stained histologic sections: months 10-36.
- Characterize changes in proliferation rates in transgenic mice by Northern and Western analysis: months 12-36.

Characterize Krct expression during proliferation in HC11 cell (months 8-16). To determine Krct's role in proliferation, Krct mRNA expression levels in actively growing, quiescent, and synchronized cells have been determined. Krct is expressed at similar levels in actively growing cells and confluent cells in two different mammary epithelial cell lines, HBI2 and 16MB9A cells. However, upon serum starvation in these cell lines, Krct mRNA levels significantly decrease. After refeeding, Krct levels remain low until just after Cyclin A induction where Krct expression dramatically increases (Fig. 1). Despite the observation that Krct mRNA levels don't change when cells become quiescent, its change in expression upon serum starvation/release demonstrates Krct is likely a cell cycle regulated gene. Additional experiments need to be performed to follow up this observation. Since Krct expression levels do not peak during this time course, serum starve/refeed experiments need to be performed again and cells harvested at later times.

Generate antisera to Krct (months 1-14). Since Krct regulation is likely to occur at the translational level due to upstream AUG codons in the 5'-untranslated region (UTR) in its mRNA, it will therefore be important to determine Krct protein levels during the cell cycle. To facilitate this analysis, antisera were generated to two different regions of the protein. GST-Krct fusion constructs were generated to the middle and C-terminal regions of Krct. These constructs were expressed in E. coli and purified using glutathione sepharose beads and cleaved with a peptidase supplied by the manufacturer. The liberated and purified Krct peptides were injected into two rabbits each. The resulting antisera were then affinity purified and tested for its ability to recognize Krct protein by Western analysis and its ability to immunoprecipitate (IP) Krct. All four antisera generated were able to detect Krct by Western analysis and IP Krct (Fig. 2). With these antisera, we will be able to determine if protein levels are cell cycle regulated similar to Krct mRNA.

Generate pTetO.Krct construct (months 1-7). Since most genes regulated by the cell cycle are themselves regulators of the cell cycle, over-expression studies will be performed to determine if Krct over-expression perturbs the cell cycle and therefore alters proliferation rates. To inducibly over-express Krct, the tetracycline-inducible plasmid-based expression system will be used. A modified Krct cDNA was generated for over-expression. PCR-based truncation of the 5'UTR was performed to remove all upstream AUG codons and place an in-frame stop codon upstream of the initiation codon. This modified cDNA was subcloned into a plasmid between

the TetO regulatory sequence and an SV40 3"UTR containing an intron for more efficient mRNA expression in mammalian cells. This expression construct will be used to generate inducible, stable clones of Krct for proliferation and differentiation assays.

Create MMTV.Krct transgenic construct (months 1-6) and create founder mice for transgenic construct (months 7-10). To further study Krct's potential role in proliferation, transgenic animals are to be generated with full length Krct cDNA placed under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter. The MMTV-Krct transgenic construct has been created and injected into oocytes from FVB mice. Injections were performed by the University of Pennsylvania Transgenic Mouse Core Facility. Southern analysis of mice generated confirmed four transgene positive animals, two females and two males. One female animal died in the process of breeding these transgene positive animals with wild type FVB mice to verify germline transmission of the transgene in these four potential founders. Autopsy analysis of this female shows her to have had only one kidney and to have been pregnant at the time of death. Presumably the stress from the pregnancy on her only kidney is what resulted in her death, not an effect from the transgene. The other three transgene positive animals are able to pass the transgene. The nest step in analysis is to determine the expression levels of mice derived from each of these lines.

Task2: Determine the role played by Krct in differentiation: months 1-36.

- Characterize Krct during HC11 cell differentiation: months 8-17.
- Perform Northern and Western analysis to detect changes in H51 and B-casein levels: months 18-26.
- Assess Krct overexpression on differentiation *in vivo*: months 10-36.
- Characterize changes in differentiation in transgenic mice by Northern and Western analysis: months 10-36.
- Characterize changes in differentiation in transgenic mice by *in situ* hybridization, whole mount analysis, and hematoxylin and eosin stained histologic sections: months 10-36.

Characterize Krct during HC11 cell differentiation (months 8-17). Over-expression of protein kinases has been shown to affect differentiation of the mammary gland. To investigate the role of Krct in differentiation, we will Krct expression during HC-11 differentiation, a mammary epithelial cell line which will express b-casein when stimulated with prolactin, dexamethasone, and insulin. Protein extracts and RNA samples have been made in the lab. Cells were stimulated with 1ug/ml of both prolactin and dexamethasone and 10% fetal calf serum. Cells were harvested for RNA and protein at 0 hours while confluent and actively growing, and 6hrs, 1,2,3,4,5,7,and 10 days post induction. Cells were all harvested during the same passage. Krct expression will be determine by Northern and Western analysis.

Once the expression profile of Krct is determined during the normal differentiation of HC-11 cells, the affects of Krct over-expression on HC-11 differentiation will be examined using the same inducible system described in Task 1. Additionally, affects of over-expression of Krct in vivo will be determined using the same MMTV-Krct transgenic animals created for Task1.

Task3: Determine the potential role played by a novel serine/threonine kinase in carcinogenesis in the mammary gland: months 1-36.

- Generate and characterize 3T3 fibroblast cell lines containing pTetO.Krct and pTetO.LacZ constructs: months 7-14.
- Perform focus formation study: months 15-22.
- Perform soft agar experiment: months 15-22.
- Determine the effects of Krct overexpression on carcinogenesis by injecting nude mice: month 12
- Characterize changes in tumor status of nude mice by gross dissection, and hematoxylin and eosin stained histologic sections: months 10-36.
- Characterize changes in tumor status of transgenic mice by gross dissection, and hematoxylin and eosin stained histologic sections: months 7-36.

Generate and characterize 3T3 fibroblast cell lines containing pTetO.Krct and pTetO.LacZ constructs: months 7-14. Several kinases have been demonstrated to function as oncogenes in human cancers and murine animal models of mammary gland carcinogenesis(11, 13-16). To investigate if Krct has a role in mammary gland carcinogenesis, we will use the inducible constructs to be generated in Task 1. These inducible cells will be tested for their ability to form foci, grow in soft agar and form tumors in nude mice. Since reagents to be used for this task were previously described in Task 1 and no further work in this task has been completed, there is nothing to report for Task 3 at this date. The generation of Krct stable clones will be performed in the near future according to schedule.

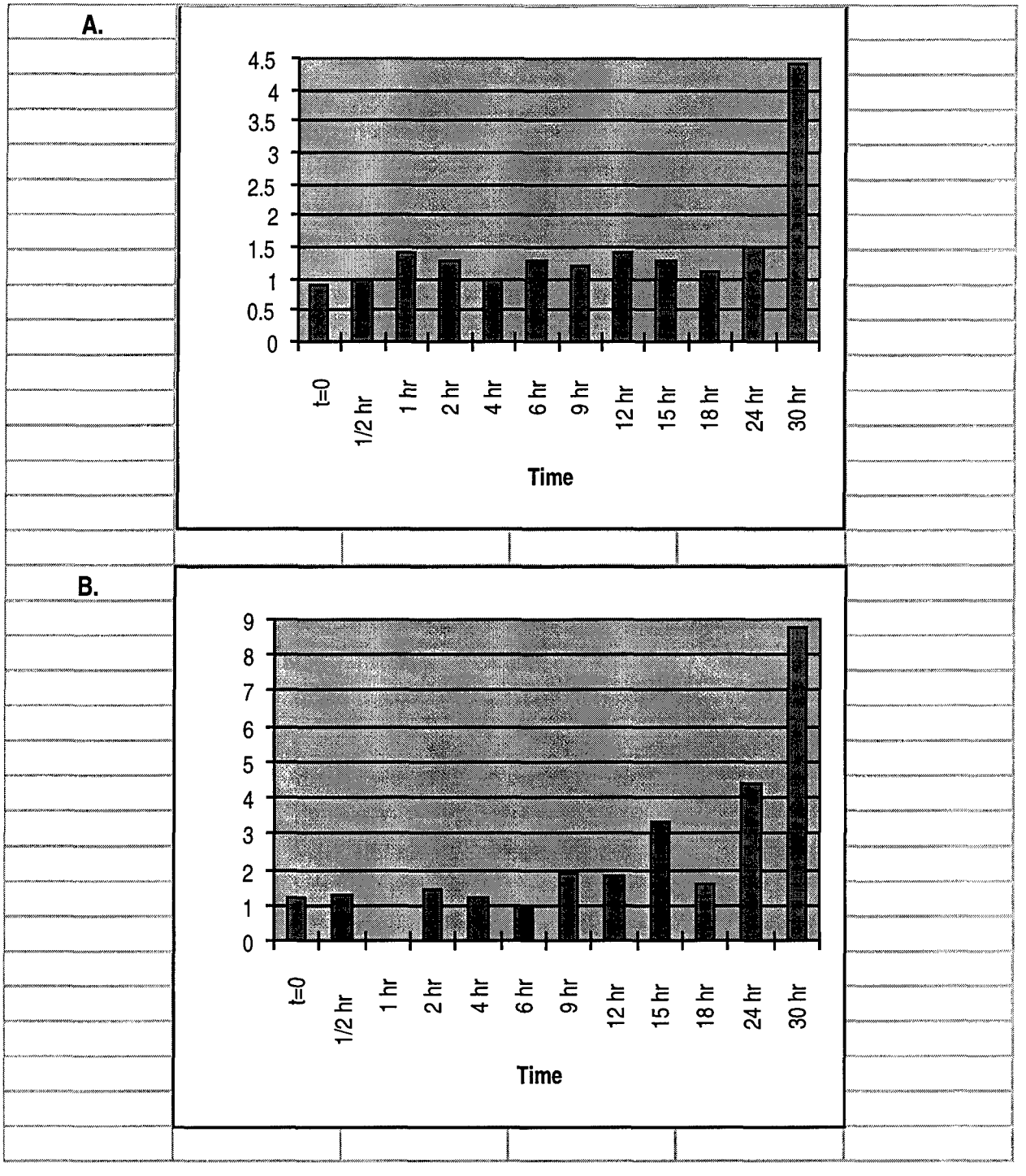

Figure 1. Krct Cell Cycle Analysis. Cells in both experiments were starved for 48 hours and then refed in media containing 10% serum. Cells were harvested at the various times indicated. Krct expression was normalized using ARPP-PO **(A)** Krct expression in HBI2 cells. Krct expression increases at 30 hours. **(B)** Krct expression in 16MB9A cells. Krct expression increases at 24 hours.

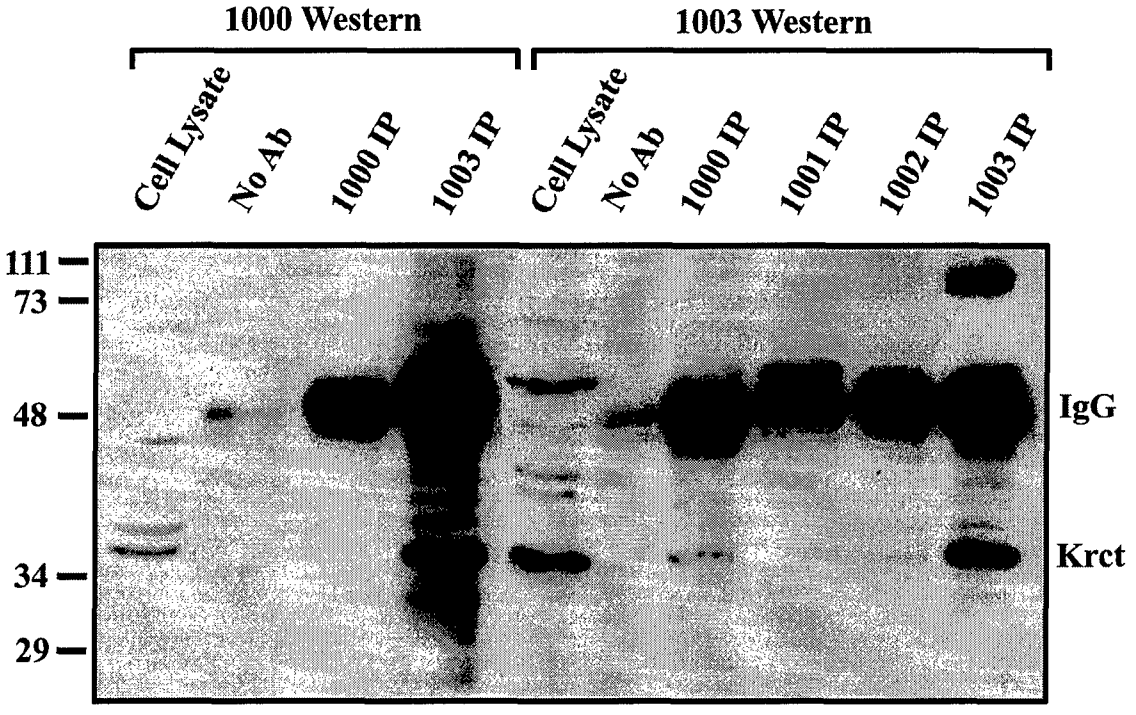

Figure 2. Krct Immunoprecipitation (IP) and Western Analysis. IP's were performed with each of the four antisera raised against Krct peptides (1000, 1001, 1002, 1003). All IP's performed used 2ug of affinity purified antisera and 500ug of cell lysate. Western analysis was performed with antisera 1000 and 1003 on the indicated samples. Both antisera recognize Krct in cell lysates. 1000, 1002, and 1003 antisera can IP Krct protein while the 1003 antisera appears to IP more Krct protein than does 1000 or 1002.

Key Research Accomplishments:

- Generated antisera to two independent regions of Krct which recognize Krct by Western analysis and Immunoprecipitation.

- Generated the TetO-Krct expression construct responsive to rtTA and tetracycline.

- Generated the MMTV-Krct expression construct for over-expression of Krct in the mammary gland of transgenic mice.

- Determined that Krct mRNA levels are regulated during the cell cycle in response to serum starvation/refeeding in two mammary epithelial cell lines.

- Generated three lines of mice carrying an MMTV-Krct transgene.

Reportable Outcomes:

(1) Stairs DB, Perry Gardner H, Ha SI, Copeland NG, Gilbert DJ, Jenkins NA, Chodosh LA. Cloning and characterization of Krct, a member of a novel subfamily of serine/threonine kinases. *Hum Mol Genet* (1998) 7(13):2157-2166.

(2) Chodosh LA, D'Cruz CM, Gardner HP, Ha SI, Marquis ST, Rajan JV, Stairs DB, Wang JY, Wang M. Mammary gland development, reproductive history, and breast cancer risk. *Cancer Res* (1999) 59(7 Suppl):1765-1771s; discussion 1771s-1772s.

Conclusions:

Analysis of Krct expression using proliferation and differentiation assays as well as determining what affects over-expression of Krct has on these processes will yield valuable insight into Krct's potential role in mammary gland development and carcinogenesis. Preliminary results from analysis of Krct expression during the cell cycle demonstrate that Krct mRNA levels are regulated. Krct expression in the serum-starved state is low compared to actively dividing cells. Upon refeeding serum-starved cells, Krct expression rises dramatically just after Cyclin A induction in the cell cycle occurs. This finding, while suggestive of a possible role for Krct in proliferation, is not conclusive. Follow-up experiments to determine if Krct protein levels are regulated in a similar manner will verify the results seen with Krct mRNA. Additionally, if Krct is a regulator of the cell cycle – as are many proteins whose expression is regulated during the cell cycle, over-expression of Krct may cause alterations in the cell cycle. Depending upon what cell cycle perturbations are seen upon over-expression of Krct, clues to the function of Krct may be gained.

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Cloning and characterization of Krct, a member of a novel subfamily of serine/threonine kinases

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Protein kinases frequently play key roles in the normal regulation of growth and development in eukaryotic organisms. As a consequence, aberrant expression or mutations in this family of molecules frequently result in transformation. Previously, we have conducted a screen to identify protein kinases that are expressed in the mouse during mammary gland development and in breast cancer cell lines. We now describe the molecular cloning, characterization and expression of Krct, a novel serine/threonine protein kinase unrelated to previously defined families of protein kinases. At the mRNA level, *Krct* is widely expressed throughout murine development and in adult tissues. Despite its ubiquitous expression, *Krct* is expressed preferentially within specific cellular compartments in multiple tissues, in particular within the testis and gastrointestinal tract. At the amino acid level, Krct is most closely related to four previously undescribed kinases in *Saccharomyces cerevisiae*, *Arabidopsis thaliana* and *Caenorhabditis elegans*. Together, these kinases appear to define a novel subfamily of serine/threonine protein kinases. *Krct* possesses an unusually long 5′-untranslated region containing multiple upstream initiation codons and, in this regard, is similar to many proto-oncogenes that regulate normal growth and differentiation. In addition, *Krct* is located on mouse chromosome 11 closely linked to the epidermal growth factor receptor and, therefore, is likely to be co-amplified in a variety of human tumors.

INTRODUCTION

Epidemiologic evidence strongly suggests that breast cancer risk is intrinsically related to the manner in which the breast normally develops during fetal life, puberty and pregnancy (1–3). The close relationship between development and carcinogenesis in the mammary gland is exemplified by the biological properties of protein kinases. Studies in a variety of model systems have demonstrated that members of this family of regulatory molecules frequently modulate the normal growth, differentiation and development of eukaryotic organisms (4). As would be predicted from this, alterations in the regulated expression or function of protein kinases frequently have been found to result in cellular transformation and neoplasia (4–7). Indeed, several members of the protein kinase family have been shown to contribute to the development of breast cancer both in humans and in rodent model systems, including c-erbB-2/Neu, the epidermal growth factor (EGF) receptor, the insulin-like growth factor 1 (IGF1) receptor, the fibroblast growth factor (FGF) receptor family, Met and c-Src (8–12). Consistent with this, overexpression of an activated form of c-erbB2/Neu and of several other protein kinases in transgenic mice results in malignant transformation of the mammary epithelium. Moreover, some protein kinases have been shown to provide prognostic information relevant to clinical outcome and response to chemotherapy. In particular, amplification of c-*erbB2/Neu* in primary breast cancers has been reported to correlate with an aggressive tumor phenotype and a poor clinical prognosis (13–17).

To explore the role played by protein kinases in development and carcinogenesis in the mammary gland, we previously performed an RT–PCR-based screen to isolate cDNA fragments of protein kinases expressed during mammary gland development and in breast cancer cell lines. A total of 43 kinases were identified, three of which have not been described previously. One of the three cDNA fragments isolated encodes a portion of a novel protein kinase, Krct (kinase related to cerevisiae and thaliana), whose identification defines a novel subfamily of serine/threonine kinases within which Krct represents the first member to be identified in vertebrates. Here we describe the cloning, expression and initial characterization of murine Krct.

RESULTS

In order to identify molecules involved in regulating mammary gland development and carcinogenesis, we used a degenerate PCR approach to amplify catalytic subdomains of protein kinases expressed in breast cancer cell lines and in the mammary gland

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during its development. Poly(A)$^+$ RNA was prepared from murine mammary glands harvested at different developmental stages as well as from three non-transformed murine mammary epithelial cell lines and four breast cancer cell lines derived from different transgenic mouse models of breast cancer. First-strand cDNA from each of these sources was prepared independently and amplified using two sets of degenerate PCR primers encoding amino acid motifs within subdomains VIb and IX that are highly conserved among protein kinases (18–20). PCR products from each cDNA source were subcloned individually and screened by a combination of DNA sequencing and colony lift hybridization. Screening of ~1500 cDNA clones using this approach resulted in the identification of 43 protein kinases, including a single clone containing a 215 bp cDNA fragment, referred to as *Bstk2*, from the catalytic domain of a novel putative serine/threonine kinase (L.A. Chodosh *et al.*, in preparation). This cDNA fragment was isolated from amplified cDNA prepared from the mammary glands of mice that had undergone a single pregnancy followed by 21 days of lactation and 2 days of post-lactational regression.

Isolation of cDNA clones encoding Krct

In order to isolate a full-length cDNA clone encompassing the catalytic domain fragment *Bstk2*, this initial 215 bp RT–PCR product was used to screen cDNA libraries prepared either from murine mammary glands at day 7 of pregnancy, or from AC816, a mammary epithelial cell line derived from an adenocarcinoma arising in an MMTV–H-ras transgenic mouse. A total of 13 cDNA clones were obtained containing inserts ranging from 1.1 to 2 kb in size. The nucleotide sequence of clones M1-1 and M2A from the mammary gland cDNA library and clones D1 and G3 from the AC816 cDNA library was determined by automated sequencing of both strands. The composite 1512 nucleotide cDNA clone encoding Krct contains the entire 215 bp RT–PCR fragment *Bstk2* from position 743 to 957. This clone also contains an open reading frame (ORF) of 915 nucleotides beginning with an AUG at nucleotide 291 (Fig. 1). Comparison of the nucleotide sequence surrounding this putative initiation codon with the Kozak consensus sequence, GCC^A/$_G$CCAUGG, reveals matches at each of the most highly conserved positions: –6, –4, –3 and +4 (21,22). Multiple stop codons are present in all three frames upstream of the putative initiation codon. These data suggest that the initiation codon of *Krct* is the AUG at nucleotide 291. The 5′-untranslated region 5′-UTR of *Krct* is unusually long, relatively GC-rich (67%), and contains three upstream AUG codons, the closest of which is located 14 nucleotides upstream of the putative initiation codon. This upstream AUG initiates an ORF of 40 amino acids that overlaps the main *Krct* ORF. The 3′-UTR is 306 nucleotides in length and contains a polyadenylation signal and poly(A) tract. Additional cDNA clones were isolated containing small insertions and deletions within the coding region, the significance of which currently is under study.

The conceptual ORF of *Krct* comprises 305 amino acids and can be divided into an N-terminal domain of 19 amino acids, a 276 amino acid putative catalytic domain, and a 10 amino acid C-terminal domain. Each of the amino acids known to be invariant among protein kinases is present in the putative catalytic domain of Krct (23). Among the sequence elements that distinguish tyrosine kinases from serine/threonine kinases, Krct is most similar to the latter, in particular with regard to the LKPXN motif in subdomain VIb and the APE motif in subdomain VIII (Fig. 1). In addition, several amino acids in subdomains I, VII, VIII, X and XI that are conserved in tyrosine kinases are absent from the *Krct* ORF.

In order to confirm the coding potential of the *Krct*, *in vitro* transcription/translation (IVT) of the full-length cDNA clone was performed. This generated a predominant 35 kDa labeled polypeptide species, consistent with the predicted 34.4 kDa size of the protein (Fig. 2). Deletion of the entire 291 nucleotide 5′-UTR containing all three upstream AUGs resulted in the same 35 kDa labeled polypeptide. These observations strongly suggest that the predicted initiation codon at nucleotide 291 is indeed the site of translation initiation. Programing of reticulocyte lysates with the full-length *Krct* cDNA also generated two lesser species at 45 and 27 kDa. These species were also generated by IVT of a 5′-UTR-deleted form of *Krct*, demonstrating that these polypeptides do not result from translation initiation at upstream AUGs followed by readthough into the *Krct* ORF.

Homology to previously isolated protein kinases

Alignment of the predicted *Krct* ORF with GenBank sequences reveals that *Krct* displays highest homology to a small group of unpublished kinases found in lower eukaryotes. The two kinases demonstrating highest homology to *Krct* are atpk3 (GenBank accession no. U97568), a serine/threonine kinase of unknown function identified in *Arabidopsis thaliana*, and YPL236c (GenBank accession no. Z73592), a hypothetical protein recognized as a kinase following analysis of the complete *Saccharomyces cerevisiae* DNA sequence (24,25). Amino acid alignment of Krct to YPL236c and atpk3 reveals 35 and 34% overall identity to *Krct*, respectively, with significant homology over the length of the Krct N-terminal and kinase catalytic domains (Fig. 3A). In comparison, amino acid alignment of the catalytic domain of Krct to those of serine/threonine kinases from other kinase families in *S.cerevisiae* such as MekI and CaMKII reveals levels of identity on the order of 17–21%. YPL236c has no residues C-terminal to the catalytic domain, and atpk3 has only four, preventing analysis of similarities to Krct in this region. Krct also displays significant homology to a putative cyclin G-associated kinase identified in *A.thaliana* (GenBank accession no. AC003033), and to a putative serine/threonine protein kinase, D2045.7, identified in *Caenorhabditis elegans* (GenBank accession no. Z35639). Alignment of the C-terminus of the putative cyclin G-associated kinase to Krct reveals that four of the 10 C-terminal residues of Krct are conserved. Placement of Krct and its most closely related kinases into an evolutionary tree on the basis of amino acid similarities within their catalytic domains demonstrates that these kinases are more closely related to each other than to any previously described group of kinases (Fig. 3B). This suggests that Krct, YPL236c, atpk3, D2045.7 and AC003033 represent a novel subfamily of protein kinases, of which Krct is the first member identified in vertebrates.

Krct encodes a functional protein kinase

In order to demonstrate that *Krct* encodes a functional kinase, a fusion protein consisting of the entire coding sequence of *Krct* fused to glutathione-*S*-transferase (GST) was generated. *In vitro* kinase assays were performed with purified recombinant GST–Krct fusion protein using histone H1 and myelin basic protein (MBP) as substrates (Fig. 4). GST-fused Krct was able to phosphorylate both histone H1 and MBP *in vitro*. Autophospho-

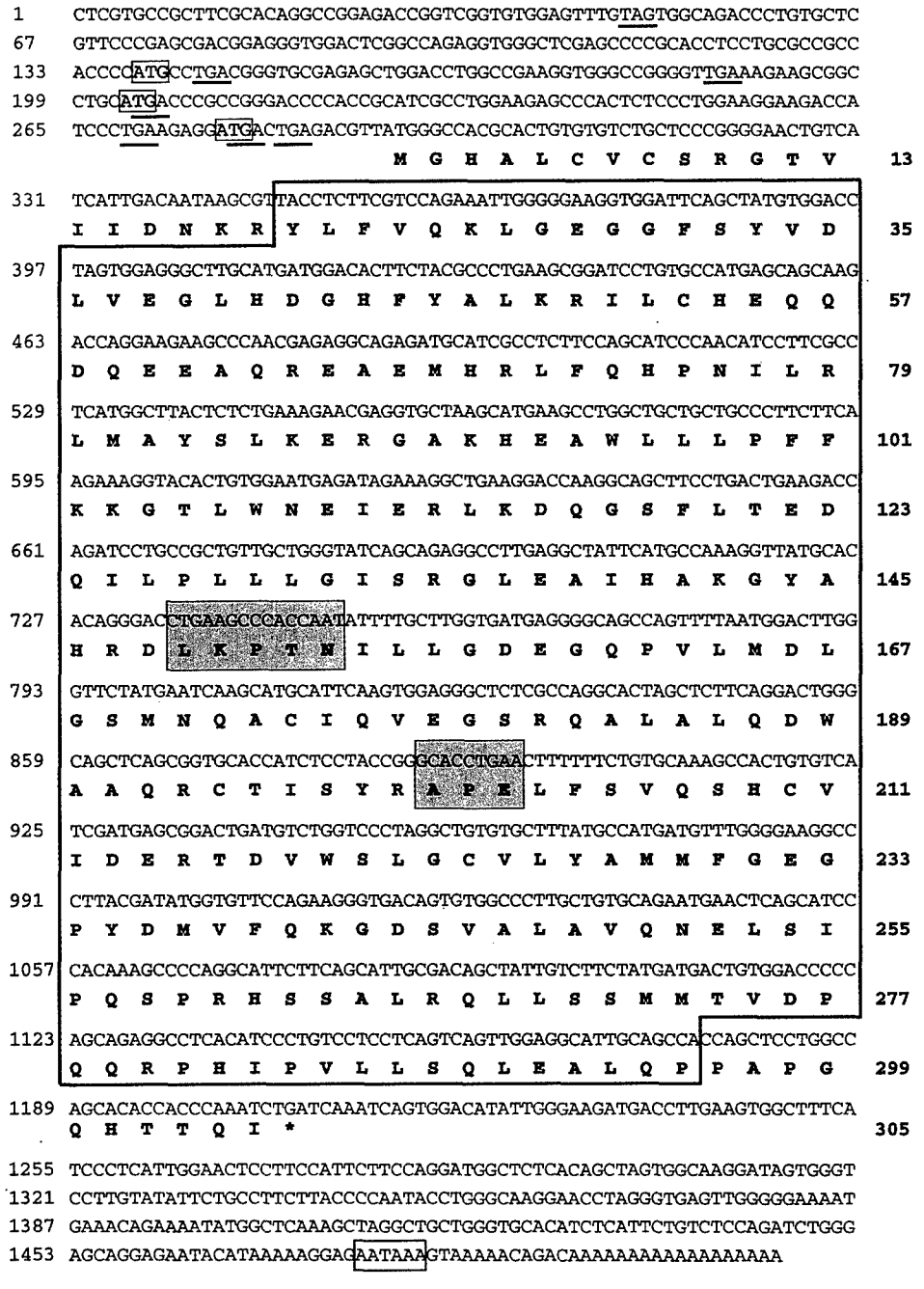

Figure 1. Nucleotide/amino acid sequence of *Krct*. The composite nucleic acid sequence and conceptual translation for *Krct* is shown. Nucleotide coordinates are shown on the left. Amino acid coordinates are shown in bold on the right. A light shaded box indicates the putative catalytic domain. Dark shaded boxes denote amino acid motifs characteristic of serine/threonine kinases. Upstream AUGs located in the 5′-UTR are indicated by shaded boxes. Stop codons present in the 5′-UTR and the polyadenylation sequence in the 3′-UTR are underlined. An asterisk denotes the stop codon for the *Krct* ORF.

rylation of the GST–Krct fusion protein was also observed. These findings confirm that *Krct* encodes a functional protein kinase.

Chromosomal localization

The mouse chromosomal localization of *Krct* was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J × *Mus spretus*)F_1 × C57BL/6J] mice (26). This interspecific backcross mapping panel has been typed for >2700 loci that are well distributed among all the autosomes as well as the X chromosome. C57BL/6J and *M.spretus* DNAs were digested with several enzymes and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms (RFLPs) using a mouse cDNA *Krct* probe. The 5.0 kb *Hin*cII and 2.5 kb *Bam*HI *M.spretus* RFLPs (see Materials and Methods) were used to follow the segregation of the *Krct* locus in backcrossed mice. The mapping results indicated that

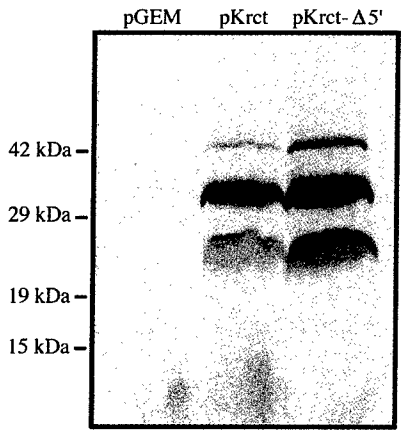

Figure 2. *In vitro* transcription/translation of *Krct*. [^{35}S]methionine-labeled Krct protein was generated using rabbit reticulocyte lysates programed with 500 ng of full-length *Krct* cDNA or 500 ng of p*Krct*-Δ5′, lacking the 291 nucleotide 5′-UTR. pGem11Zf plasmid was used as a control. The relative migration of molecular weight markers is indicated.

Krct is located in the proximal region of mouse chromosome 11 linked to *Ikaros*, *Egfr* and *Rel*. Although 125 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 5), up to 174 mice were typed for some pairs of markers. Each locus was analyzed in pairwise combinations for recombination frequencies using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are: centromere–*Ikaros* (1/174)–*Egfr* (0/164)–*Krct* (6/133)–*Rel*. The recombination frequencies [expressed as genetic distances in centiMorgans (cM) ± standard error] are *Ikaros* (0.6 ± 0.6)–(*Egfr*, *Krct*) (4.5 ± 1.8). No recombinants were detected between *Egfr* and *Krct* in 164 animals typed in common, suggesting that the two loci are within 1.8 cM of each other (upper 95% confidence limit).

We have compared our interspecific map of chromosome 11 with a composite mouse linkage map that reports the map location of many uncloned mouse mutations (provided by the Mouse Genome Database, a computerized database maintained at the Jackson Laboratory, Bar Harbor, ME). *Krct* mapped in a region of the composite map that lacks mouse mutations with a phenotype that might be expected for an alteration in this locus (data not shown). The proximal region of mouse chromosome 11 shares homology with human chromosomes 7p and 2p (summar-

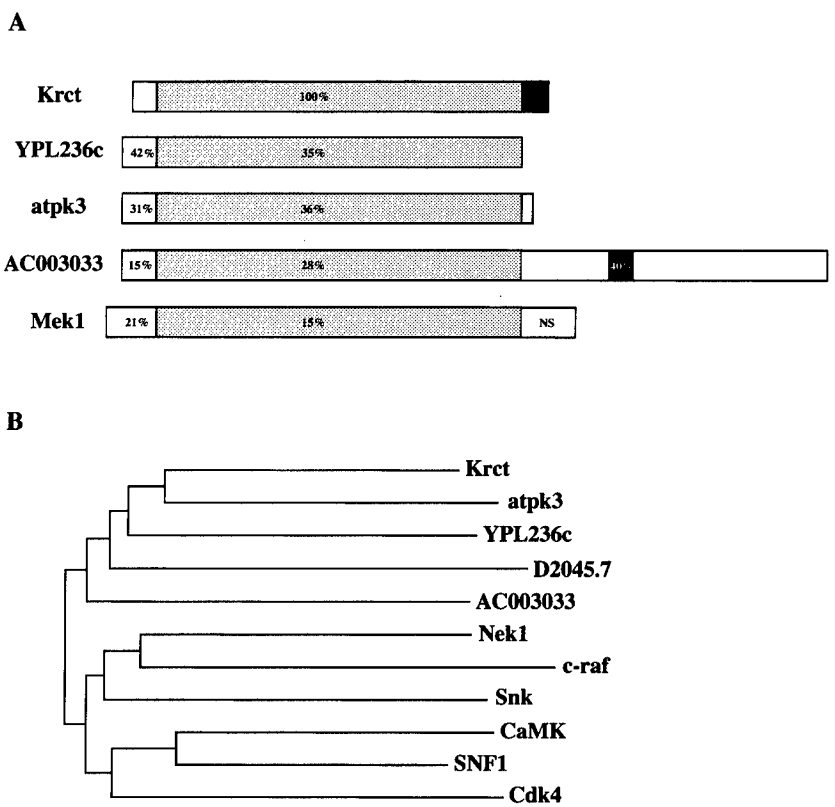

Figure 3. *Krct* defines a novel subfamily of serine/threonine kinases. (**A**) Schematic representation of amino acid homology between Krct, YPL236c, atpk3 and AC003033. Results of the pairwise analysis using the ClustalW alignment program of the N-terminus, catalytic domain and C-terminus of Krct, YPL236c, atpk3, AC003033 and Mek1 are listed. The percentage identity to Krct within each of these domains is indicated for each kinase. N-terminal, catalytic and C-terminal domains are indicated by open boxes, shaded boxes and black boxes, respectively. NS, not significant. (**B**) Phylogenetic tree illustrating the relationship of Krct with other serine/threonine kinases in the GenBank database. Analysis and depiction of results was performed using the ClustalX multisequence alignment program and DendroMaker 4.0.

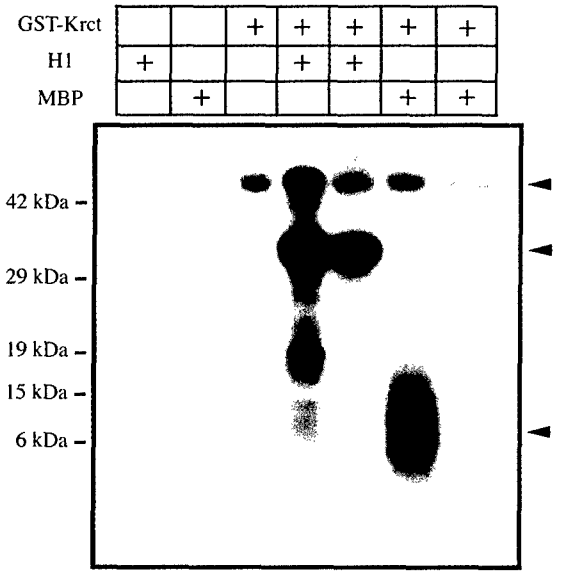

Figure 4. *Krct* encodes a functional protein kinase. Histone H1 and myelin basic protein (MBP) were used as *in vitro* kinase substrates for purified GST–Krct fusion protein. Histone H1 and MBP were incubated in the absence or presence of purified GST–Krct, as indicated. Reactions were performed using either 2 μg (lanes 3, 4 and 6) or 7 μg (lanes 5 and 7) of GST–Krct. Arrowheads indicate the relative migration of GST–Krct (top), histone H1 (middle) and MBP (bottom). The relative migration of molecular weight markers is indicated.

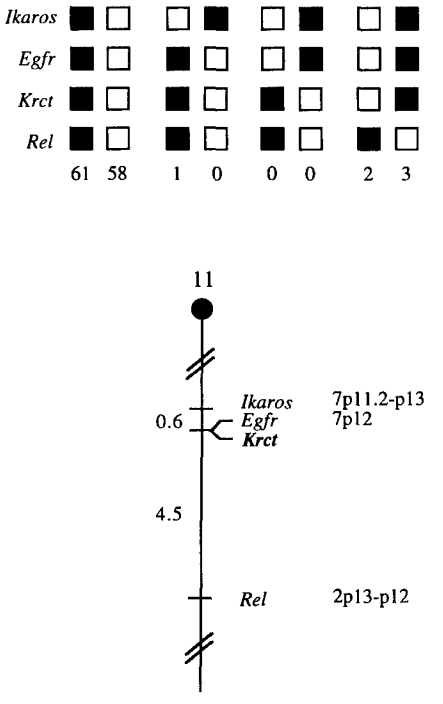

Figure 5. *Krct* maps in the proximal region of mouse chromosome 11. *Krct* was placed on mouse chromosome 11 by interspecific backcross analysis. The segregation patterns of *Krct* and flanking genes in 125 backcrossed animals that were typed for all loci are shown at the top of the figure. For individual pairs of loci, >125 animals were typed (see text). Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *M.spretus*) F_1 parent. The shaded boxes represent the presence of a C57BL/6J allele and white boxes represent the presence of an *M.spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. A partial chromosome 11 linkage map showing the location of *Krct* in relation to linked genes is shown at the bottom of the figure. Recombination distances between loci in cM are shown to the left of the chromosome, and the positions of the loci in human chromosomes, where known, are shown to the right. References for the human map positions of loci cited in this study can be obtained from the Genome Data Base, a computerized database of human linkage information maintained by The William H. Welch Medical Library of the Johns Hopkins University (Baltimore, MD).

ized in Fig. 5). In particular, *Egfr* has been mapped to 7p12. The close linkage between *Egfr* and *Krct* in mouse suggests that the human homolog of *Krct* will map to 7p as well.

Analysis of *Krct* mRNA expression

In order to begin to analyze the biological role played by *Krct*, the spatial and temporal pattern of mRNA expression of this gene was determined during fetal development and in adult tissues in the mouse. Northern hybridization analysis of RNA isolated from FVB embryos at embryonic days E6.5, E13.5 and E18.5 using a *Krct* cDNA probe revealed an abundantly expressed 1.5 kb mRNA species at each of these developmental time points (Fig. 6A). The size of this message matches that predicted based on the size of the isolated cDNA clones, suggesting that the 1512 nucleotide *Krct* cDNA sequence represents the full-length message. *In situ* hybridization performed on E13.5 and E18.5 embryos using an antisense *Krct* cDNA probe revealed widespread *Krct* expression in most tissues of the developing mouse, with particularly high levels of expression observed in the fetal liver, dorsal root ganglia and cortical regions of the kidney at E13.5 and E18.5 (Fig. 6B). High levels of *Krct* expression were also observed in salivary gland, brown adipose tissue and hair follicles at E18.5.

Krct expression was also determined at the mRNA level in tissues of the adult mouse. Northern hybridization revealed that *Krct* is expressed in all organs examined, with highest levels of expression present in the mammary gland, ovary, liver, kidney and small intestine (Fig. 7). *In situ* hybridization analysis of *Krct* mRNA expression revealed that although *Krct* is widely expressed in tissues of the adult mouse, *Krct* expression within these tissues occurs preferentially in certain cell types. In the gastrointestinal tract, *Krct* is highly expressed in epithelial compartments of the stomach, duodenum and colon, and is expressed at significantly lower levels in the mesenchymal compartments of these tissues (Fig. 8). Similarly, *Krct* is expressed at higher levels in the glandular epithelium of the prostate than in its stromal component. In the mammary gland, *Krct* is expressed at high levels in the alveolar and ductal epithelium and at moderate though somewhat lower levels in the stroma. In the testis, *Krct* is expressed at high levels in the peripheral regions of seminiferous tubules that are populated principally by Sertoli cells, spermatogonia and primary spermatocytes. *Krct* is expressed at lower levels in Leydig cells within the interstitial compartment of the testis and in the central region of seminiferous tubules populated by the more differentiated spermatids and spermatozoa. All regions of the brain were found to express *Krct*, although expression was particularly high in the hippocampus and dentate gyrus (Fig. 9).

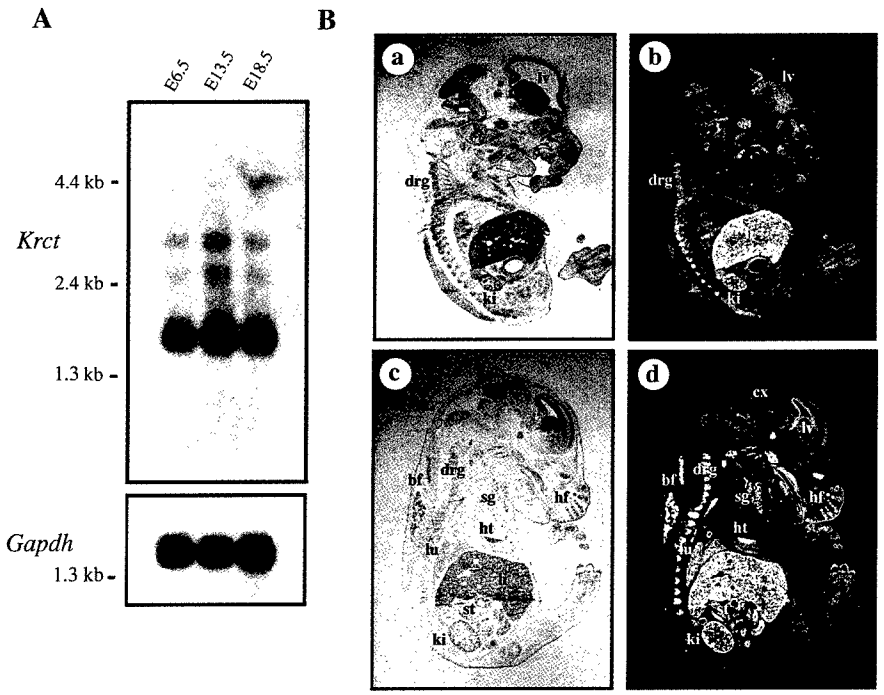

Figure 6. Expression of *Krct* during murine embryogenesis. (**A**) Northern hybridization analysis of 1 μg of poly(A)$^+$ RNA from day E6.5, E13.5 and E18.5 embryos hybridized with a cDNA probe specific for *Krct*. The blot was stripped and reprobed with *Gapdh* as a loading control. The relative migration of RNA size markers is indicated. (**B**) *In situ* hybridization analysis of *Krct* mRNA expression. Bright-field (a and c) and dark-field (b and d) photomicrographs of E13.5 (a and b) and E18.5 (c and d) FVB embryo sections hybridized with a *Krct* antisense cDNA probe. No signal over background was detected in serial sections hybridized with sense *Krct* probes. Magnification: 8× (a and b); 6.3× (c and d). bf, brown adipose tissue; cx, cortex; drg, dorsal root ganglia; hf, hair follicles; ht, heart; ki, kidney; li, liver; lu, lung; lv, lateral ventricle; sg, salivary gland; st, stomach.

DISCUSSION

The novel serine/threonine kinase, Krct, was isolated initially as a cDNA fragment in a degenerate oligonucleotide PCR screen designed to identify protein kinases potentially involved in mammary gland development and carcinogenesis. Herein we describe the molecular cloning and analysis of a full-length murine cDNA clone encoding *Krct*. *Krct* is widely expressed in the mouse at the mRNA level and encodes a 35 kDa protein with proven phosphotransferase capabilities. Amino acid sequence analysis indicates that Krct is most closely related to a group of unpublished kinases identified in *S.cerevisiae*, *A.thaliana* and *C.elegans* of unknown function. Comparison of the amino acid sequence of this group of genes with those from each of the major branches of the kinase superfamily suggests that these genes represent a novel subfamily of serine/threonine protein kinases, of which *Krct* represents the first member to be described in vertebrates.

Krct is expressed in the mouse in a broad range of tissues and at high levels throughout fetal development as well as in the adult mouse. In contrast to the widespread expression of *Krct* as determined by northern hybridization, *in situ* hybridization analysis reveals that *Krct* is expressed preferentially in specific cell types within these tissues. In particular, *Krct* is expressed preferentially in epithelial as compared with mesenchymal compartments of the prostate, stomach, duodenum, colon and, to a lesser extent, mammary gland. Similarly, *Krct* is expressed in the testis and brain in a cell type-specific pattern, being expressed at the highest levels in the testis in spermatogonia and primary spermatocytes, and in the brain in the hippocampus and dentate gyrus.

The 5′-UTR of *Krct* is 291 nucleotides in length. Interestingly, of 346 vertebrate mRNAs studied by Kozak, only 7.8% were longer than 200 nucleotides and only 2.3% were longer than 300 nucleotides (21). Long 5′-UTRs are generally associated with poor translational efficiency and/or translational regulation (21,22). Moreover, the 5′-UTR of *Krct* contains three upstream AUG codons, the most proximal of which lies only 14 nucleotides upstream of the initiation codon and initiates an ORF with an overlapping reading frame. Excluding proto-oncogenes, <10% of vertebrate mRNAs contain upstream AUGs, and the majority of those which do contain only one (21,22). In contrast, two-thirds of proto-oncogenes as well as a high percentage of growth factors, signal transduction proteins and transcription factors contain at least one upstream AUG. The unusual length of the 5′-UTR, as well as the presence and location of upstream initiation codons, suggest that *Krct* expression may be regulated at the level of translational initiation, and are consistent with a role for *Krct* in regulating normal growth and differentiation.

Our data indicate that *Krct* is located within 1.8 cM of the *Egfr* locus on mouse chromosome 11. *Egfr* is amplified and overexpressed in a variety of human tumors including glioblastomas and squamous cell carcinomas of the head and neck. Previous studies have demonstrated that additional genes located within 2 cM of *Egfr* are also amplified and overexpressed in glioblastomas (27). These data suggest that *Krct* may be contained on the *Egfr* amplicon and may be overexpressed in human cancers. Whether

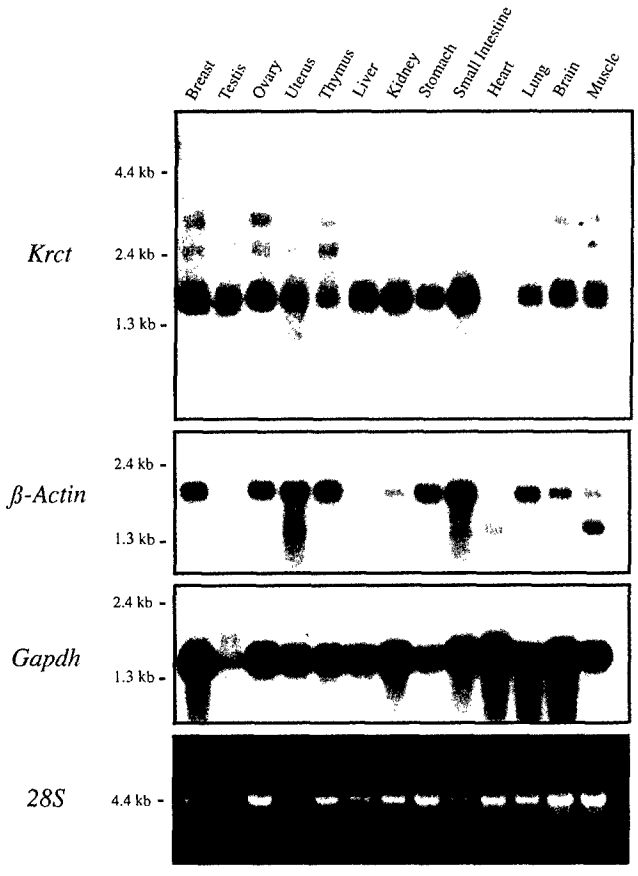

Figure 7. Northern hybridization analysis of *Krct* in tissues of the adult mouse. A northern blot containing 1 μg of poly(A)$^+$ RNA isolated from the indicated murine tissues was hybridized with a cDNA probe specific for *Krct*. The hybridization patterns for the same blot probed with a *b-actin* cDNA fragment, and a blot containing the same RNA samples probed with a *Gapdh* cDNA fragment, are shown. The 28S rRNA band is shown from the ethidium bromide-stained nitrocellulose blot.

such overexpression could contribute to the biological behavior of these tumors remains to be tested.

When considered with its cell type-specific pattern of expression, the cDNA structure and apparent conservation of this novel subfamily of protein kinases during evolution suggest that Krct may play a significant role in vital cellular processes. As such, it will be important to identify the signaling pathways in which Krct participates. In this regard, insights into these pathways may be gained through analysis of the functions of Krct family members in lower eukaryotes.

MATERIALS AND METHODS

Krct cloning

Poly(A)$^+$ RNA isolated from a H-*ras* transgenic mammary epithelial tumor cell line, AC816, and from the mammary glands of FVB mice at day 7 of pregnancy were each used to generate independent cDNA libraries in λZAP using the ZAP-cDNA synthesis kit and ZAP-cDNA Gigapack II Gold packaging kit according to the manufacturer's instructions (Stratagene). A total of 5×10^5 plaques from each library were screened by standard methods using a [^{32}P]dCTP-labeled random-primed cDNA fragment (BMB Random Prime) corresponding to nucleotides 921–1135 of *Krct*. Hybridization was performed at a concentration of 10^6 c.p.m./ml in 48% formamide, 10% Dextran sulfate, 4.8× SSC, 20 mM Tris (pH 7.5), 10× Denhart's solution, 20 μg/ml salmon sperm DNA and 0.1% SDS at 42°C overnight. Following hybridization, washes were performed in 2× SSC/0.1% SDS. Filters were washed in 2× SSC/0.1% SDS at room temperature for 30 min (×2), followed by one 30 min wash at 50°C, and subjected to autoradiography (Kodak XAR-5). A total of 13 phage clones were plaque purified and plasmids liberated by *in vivo* excision according to the manufacturer's instructions (Stratagene). The nucleotide sequence of two clones from each library was determined by automated sequencing of both strands using an ABI Prism 377 DNA sequencer. The full-length *Krct* cDNA sequence has been deposited in the GenBank database (accession no. AF089869).

Tissue preparation

FVB mice were housed under barrier conditions with a 12 h light/dark cycle. Mouse embryos and mammary gland tissue were harvested at specified time points following timed matings. Day 0.5 p.c. was defined as noon of the day on which a vaginal plug was observed. Organs were harvested from 15- to 16-week-old virgin mice. Tissues used for RNA preparation were snap-frozen on dry ice. Tissues used for *in situ* hybridization analysis were either embedded in OCT medium and frozen in a dry ice/isopentane bath or fixed in neutral buffered formalin for 24 h followed by paraffin embedding.

Northern analysis

RNA was prepared by homogenization of cultured cells or snap-frozen tissue samples in guanidinium isothiocyanate followed by ultracentrifugation through cesium chloride as previously described (28,29). Poly(A)$^+$ RNA was selected using oligo(dT) cellulose (Pharmacia). Northern hybridization was performed as described (28). Probes used for analysis were generated by random-primed labeling of cDNA fragments and included nucleotides 624–1234 of *Krct*, 1142–1241 of *b-actin* and 466–1056 of *Gapdh*. Hybridization was performed overnight at 42°C. Following hybridization, washes were performed in 2× SSC/0.1% SDS at room temperature for 30 min (×2), followed by one 30 min wash at 50°C, and subjected to autoradiography (Kodak XAR-5).

In vitro transcription/translation

In vitro transcription/translation was performed on 500 ng of DNA using rabbit reticulocyte lysates and [^{35}S]methionine according to the manufacturer's instructions (Promega). Samples were electrophoresed on a 17% SDS–PAGE gel and subjected to autoradiography. *Krct* was subcloned into pGem11Zf (Promega) by *Eco*RI and *Apa*I digestion of the *Krct* D1 clone in pBS SK(+/–) and ligation of the purified *Krct* cDNA fragment (nucleotides 11–1512) into the *Eco*RI and *Apa*I sites of pGem11Zf. Deletion of the 5′-UTR was performed by PCR-based mutagenesis. Oligonucleotide primers CTGAATTCACTGAGACGTTATGGGCCACG, containing an *Eco*RI site, and CCCCTCATCACCAAGC were used to amplify nucleotides 280–767 of the D1 *Krct* cDNA clone using *Taq* polymerase (BMB). PCR was

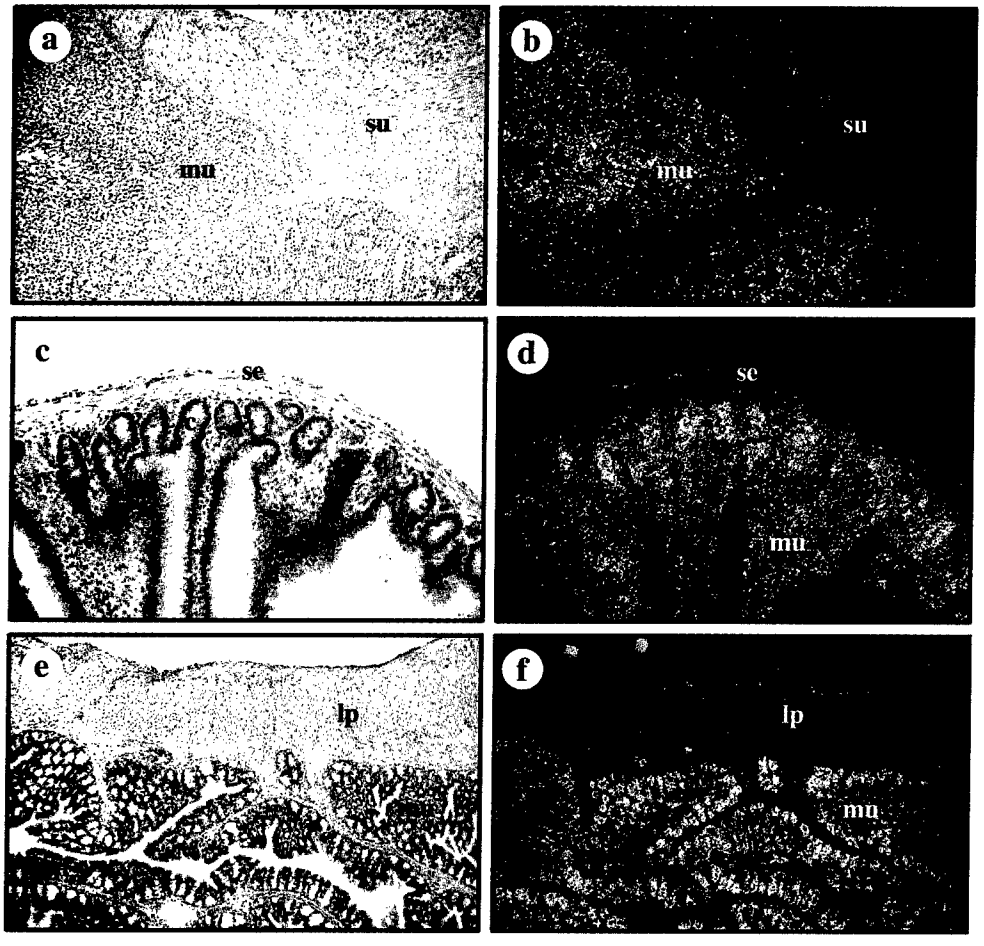

Figure 8. Spatial localization of *Krct* expression in the gastrointestinal tract. Bright-field (a, c and e) and dark-field (b, d and f) photomicrographs of *in situ* hybridization analysis performed on sections of stomach (**a** and **b**), duodenum (**c** and **d**) and colon (**e** and **f**) hybridized with a *Krct* antisense probe. No signal over background was detected in serial sections hybridized with a sense *Krct* probe. Magnification: 100× (a and b); 200× (c–f). c, intestinal crypts; lp, lamina propria; mu, mucosa; se, serosa; su, submucosa.

performed in a Perkin Elmer GeneAmp PCR System 9600 thermocycler under the following conditions: initial denaturation at 95°C for 5 min followed by 30 s at 94°C, 30 s at 55°C and 2 min at 72°C for 10 cycles. A final extension was performed at 72°C for 5 min. The PCR product generated contained an *Eco*RI restriction site at the 5′ end and an internal *Bam*HI site at nucleotide 428 of *Krct*. This *Eco*RI–*Bam*HI fragment was purified and ligated into the D1 clone in pGem11Zf following digestion with *Eco*RI and *Bam*HI to yield a 5′-deleted form of *Krct*.

Kinase assay

The full-length *Krct* ORF was subcloned in-frame in pGEX6P-1 (Pharmacia) and expressed in bacterial strain BL21 by induction with 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG) at 30°C for 3 h. Protein was purified by passage over glutathione–Sepharose 4B beads according to the manufacturer's instructions (Pharmacia). *In vitro* kinase activity was assayed under final reaction conditions consisting of 20 mM Tris (pH 7.5), 5 mM $MgCl_2$ and 100 μM ATP, using 3 μg of either histone H1 or MBP (Sigma) as a substrate, and either 2 or 7 μg of the purified bound GST–Krct fusion protein. Following a 30 min incubation at 30°C, samples were electrophoresed on a 15% SDS–PAGE gel and subjected to autoradiography.

In situ hybridization

In situ hybridization was performed as described (28). Antisense and sense probes were synthesized with the Promega *in vitro* transcription system using [^{35}S]UTP and [^{35}S]CTP from the T7 and SP6 RNA polymerase promoters of a PCR template containing sequences corresponding to nucleotides 624–1234 of *Krct*. Exposure times were: 4 weeks for OCT sections of colon, prostate, duodenum, brain and E18.5 embryo; 4 weeks 5 days for OCT sections of stomach, E13.5 embryo and mammary gland at day 7 of pregnancy; and 8 weeks 6 days for paraffin sections of testis.

Sequence analysis

Sequence analysis including predicted ORFs and calculation of the molecular weight of Krct was performed using MacVector. Pairwise sequence alignments were performed using the ClustalW alignment program. Multiple sequence alignment and

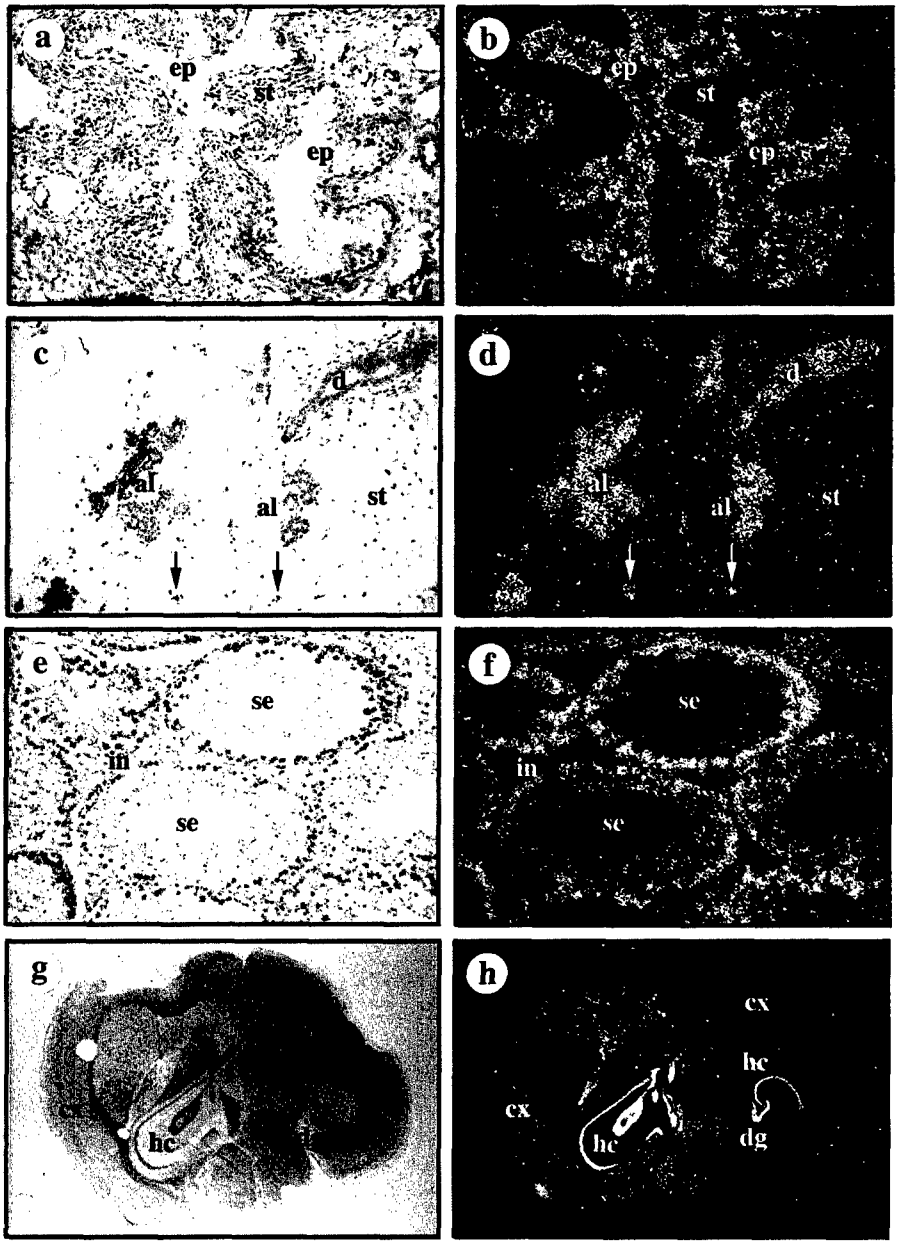

Figure 9. Spatial localization of *Krct* expression in tissues of the adult mouse. Bright-field (a, c, e and g) and dark-field (b, d, f and h) photomicrographs of *in situ* hybridization analysis performed on sections of dorsolateral prostate (**a** and **b**), number four mammary gland of a day 7 pregnant mouse (**c** and **d**), testis (**e** and **f**) and brain (**g** and **h**) hybridized with a *Krct* antisense probe. Arrows indicate *Krct*-expressing stromal cells in the mammary gland. No signal over background was detected in serial sections hybridized with a sense *Krct* probe. Magnification: 200× (a–f); 63× (g and h). al, alveoli; cx, cortex; d, duct; dg, dentate gyrus; ep; epithelium; hc, hippocampus; in; interstitium; st; stroma; se; seminiferous tubules.

phylogenetic calculations were performed using the ClustalX multisequence alignment program. DendroMaker 4.0 was used to draw a phylogenetic tree.

Interspecific mouse backcross mapping

Interspecific backcross progeny were generated by mating (C57BL/6J × *M.spretus*)F_1 females and C57BL/6J males as described. In total, 205 N_2 mice were used to map the *Krct* locus (see text for details). DNA isolation, restriction enzyme digestion, agarose gel electropheresis, Southern blot transfer and hybridization were performed essentially as described. All blots were prepared with Hybond-N^+ nylon membrane (Amersham). The probe, an ~575 bp *Eco*RI fragment of mouse cDNA was labeled with [^{32}P]dCTP using a nick translation labeling kit (BMB); washing was carried out to a final stringency of 1× SSCP, 0.1% SDS, 65°C. Fragments of 6.0 and 3.6 kb were detected in *Hinc*II-digested C57BL/6J (B) DNA, and fragments of 5.0 and 3.6 kb were detected in *Hinc*II-digested *M.spretus* (S) DNA. In addition, *Bam*HI digestion produced fragments of 5.3 and 4.6 kb (B) and 5.3 and 2.5 kb (S). The presence or absence of the

M.spretus-specific fragments, which co-segregated, was followed in the backcrossed mice. The *Hin*cII and *Bam*HI data were combined.

A description of the probes and RFLPs for the loci linked to *Krct* including *Ikaros*, *Egfr* and *Rel* have been reported previously. Recombination distances were calculated using Map Manager, version 2.6.5. Gene order was determined by minimizing the number of recombination events to explain the allele distribution patterns.

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NOTE ADDED IN PROOF

While this manuscript was under review, a kinase with nucleotide sequence nearly identical to that of Krct was described by J.M. Ligos, N. Gerwin, P. Fernandez, J.C. Gutierrez-Ramos and A. Bernad (1998) Cloning, expression analysis, and functional characterization of PKL12, a member of a new subfamily of ser/thr kinases. *Biochem. Biophys. Res. Commun.*, **249**, 380–384.

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Mammary Gland Development, Reproductive History, and Breast Cancer Risk[1]

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Abstract

The observation that normal pathways of differentiation and development are invariably altered during the process of carcinogenesis implies an intrinsic relationship between these processes. This relationship is particularly evident in the breast, as exemplified by the existence of endocrine risk factors for breast cancer that are related to the timing of normal developmental events. Understanding the mechanisms by which normal developmental events alter breast cancer risk is a central focus of our laboratory. Herein, we describe three approaches being taken in our laboratory toward defining the molecular basis of this relationship. These include: determining the roles played by the tumor suppressor genes, *BRCA1* and *BRCA2*, in the normal differentiation and development of the breast; studying the function of three novel protein kinases identified in our laboratory in mammary epithelial development; and defining the molecular and cellular changes that occur in the breast as a result of reproductive events known to influence breast cancer risk.

Introduction

A basic tenet emerging from studies in cancer biology is that normal pathways of differentiation and development are inevitably disrupted during the process of carcinogenesis. This implies an intrinsic relationship between these processes. The existence of endocrine risk factors for breast cancer that are related to the timing of normal developmental events such as menarche, menopause, and age at first full-term pregnancy epitomizes this relationship. The recognition that breast cancer risk is determined in part by the same reproductive endocrine events that drive mammary gland development argues that mammary gland development and mammary carcinogenesis are fundamentally related.

One of the most intriguing examples of this principle is the observation that women who undergo their first full-term pregnancy early in life (*i.e.*, early parity) have a significantly reduced lifetime risk of breast cancer (1). The magnitude of this parity-induced protection against breast cancer is similar in many countries and ethnic groups, regardless of endemic incidence. This suggests that protection results from an intrinsic effect of parity on the biology of the breast rather than from extrinsic factors specific to a particular environmental, genetic, or socioeconomic setting. This conclusion is bolstered by the observation that rats that have previously undergone a full-term pregnancy are resistant to the induction of breast cancer by administration of the carcinogen DMBA,[3] as compared to age-matched nulliparous controls (2, 3). Therefore, both human epidemiology and animal model systems support the conclusion that an early first full-term pregnancy results in a permanent change in the breast, either directly or indirectly, that confers a decreased risk for the subsequent development of breast cancer. Although this effect has been hypothesized to result from the impact of terminal differentiation on the susceptibility of the mammary epithelium to carcinogenesis, the molecular and cellular basis for this phenomenon is unknown.

A second illustration of this principle comes from the observation that breast cancer risk attributable to exposure to ionizing radiation is a function of age at the time of exposure. Specifically, studies of women who received mantle irradiation for Hodgkin's disease or who underwent repeated fluoroscopy in the course of treatment for tuberculosis have demonstrated that breast cancer risk is significantly greater in women who were exposed to ionizing radiation during adolescence as compared to women exposed at later ages (4, 5). Analogously, nulliparous rats fed DMBA are more likely to develop breast cancer if they are exposed during puberty rather than as mature adults (6). Interestingly, epidemiological studies suggest that the increased susceptibility of the immature human breast to early events in carcinogenesis may occur prior to as well as during puberty. Studies of survivors from Hiroshima and Nagasaki indicate that the greatest increase in breast cancer risk occurred in women who were less than 10 years old at the time of exposure (7). The observed increase in breast cancer incidence in women irradiated during the first year of life for presumed thymic enlargement is perhaps an even more impressive illustration of this principle, given the rudimentary state of the mammary gland at this age (8). Together, these studies suggest that the susceptibility of the mammary gland to carcinogenesis is related to the gland's developmental state at the time of exposure to mutagenic agents and that the immature breast is particularly susceptible to early events in carcinogenesis.

Understanding the molecular and cellular mechanisms by which normal developmental events alter breast cancer risk is a central goal of our laboratory. We believe that achieving this goal requires a more complete understanding of the manner in which hormones and reproductive history alter subpopulations of epithelial cell types present in the breast and of the roles played by key regulatory molecules in these processes. Toward this end, we are currently focusing on: (*a*) determining the roles played by the tumor suppressor genes, *BRCA1* and *BRCA2*, in the normal differentiation and development of the breast; (*b*) studying the function of three novel protein kinases identified in our laboratory in mammary epithelial development and carcinogenesis; and (*c*) defining the molecular and cellular changes that occur in the breast as a result of reproductive events known to influence breast cancer risk.

Tumor Suppressor Genes: *BRCA1* and *BRCA2*

The epidemiological relationship between development and carcinogenesis is illustrated on a molecular and mechanistic level by the existence and function of tumor suppressor genes such as *p53*, the Wilms' tumor gene (*WT1*), and the retinoblastoma susceptibility gene (*RB*). Germ-line mutations in these genes are associated with inherited cancer predisposition syndromes (9). The cloning and analysis of several tumor suppressor genes has revealed that they frequently

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[3] The abbreviations used are: DMBA, 7,12-dimethylbenz[*a*]anthracene; EGFR, epidermal growth factor receptor.

encode proteins that act as negative regulators of cell proliferation, exert cell cycle checkpoint control function, or maintain genome integrity (10, 11). In addition, the targeted deletion of these genes in mice frequently results not only in increased susceptibility to cancer but also in abnormalities in proliferation, apoptosis, differentiation, and development (10, 12). As such, one approach to elucidating the relationship between mammary gland development and carcinogenesis is to determine the function of tumor suppressor genes known to be involved in the pathogenesis of breast cancer.

Genetic analysis of families in which multiple individuals have developed breast cancer suggests that 5–10% of breast cancer cases result from the inheritance of germ-line mutations in autosomal dominant susceptibility genes (13, 14). Over the past 4 years, several of these breast cancer susceptibility genes have been isolated by positional cloning, including *BRCA1* and *BRCA2* (15–19). Tumors arising in patients with germ-line mutations in either *BRCA1* or *BRCA2* typically display loss of the corresponding wild-type allele, suggesting that *BRCA1* and *BRCA2* are tumor suppressor genes (20–22). Interestingly, *BRCA1* and *BRCA2* mutations have not been identified in sporadic breast cancers, despite the fact that 25–30% of sporadic breast cancers show loss of heterozygosity at these loci (16, 23–26). This raises the intriguing possibility that the normal functions of these genes are temporally and/or developmentally restricted.

Recently, important clues to BRCA1 and BRCA2 function have come from biochemical studies demonstrating that treatment of cells with a variety of DNA-damaging agents leads to the rapid phosphorylation of BRCA1 (27, 28). Moreover, both BRCA1 and BRCA2 have been shown to directly or indirectly bind to RAD51, a homologue of RecA that has been implicated in DNA repair and recombination (29–32). These and other observations have led to the hypothesis that BRCA1 and BRCA2 are involved in the cellular response to DNA damage. Consistent with this hypothesis, embryonic cells from mice homozygous for mutations in the *Brca2* locus have an increased sensitivity to DNA-damaging agents (30, 33, 34). It is interesting to speculate that the developmental regulation of *BRCA1* and *BRCA2* expression or function may contribute to the age-dependent susceptibility of the breast to ionizing radiation-induced carcinogenesis described above.

The markedly elevated risk of breast cancer observed in women carrying germ-line mutations in *BRCA1* and *BRCA2* strongly suggests that these gene are critical for the properly regulated growth of mammary epithelial cells. As a first step toward understanding the developmental role of *BRCA1* and *BRCA2*, we have analyzed the spatial and temporal expression of the murine homologues of these genes during embryogenesis, in the mammary gland during postnatal development, and in adult tissues (35, 36). These studies reveal that expression of both *Brca1* and *Brca2* are tightly regulated during mammary gland development. For example, *Brca1* and *Brca2* expression levels in the mammary glands of adolescent female mice undergoing ductal morphogenesis are significantly higher than those found in the mammary glands of mature females in whom ductal morphogenesis has been completed (35, 36). This temporal pattern of expression is explained in part by the observation that *Brca1* and *Brca2* are expressed at high levels in terminal end buds, which are puberty-specific structures that contain rapidly proliferating cells undergoing differentiation (35–37). *Brca1* and *Brca2* mRNA levels are also markedly up-regulated in the mammary gland early in pregnancy, a period during which alveolar buds begin the process of rapid proliferation and differentiation to form mature, milk-producing alveoli (35–38). This up-regulation of *Brca1* and *Brca2* expression occurs preferentially in developing alveoli as compared to adjacent epithelial ducts, consistent with patterns of proliferation (35, 36). Indeed, at virtually all stages of development, *Brca1* and *Brca2* expression are restricted to cellular compartments actively involved in proliferation and differentiation. These patterns of expression suggest that these tumor suppressor genes may play a role in the normal development of the breast and other tissues.

The spatial and temporal patterns of *Brca1* and *Brca2* expression during development likely reflect the fact that expression of these genes is tightly regulated as a function of proliferation. We have shown that *Brca1* and *Brca2* mRNA levels are high in exponentially growing cells and low in quiescent cells (39). During progression through the cell cycle, *Brca1* and *Brca2* mRNA levels increase during G_1 and attain maximal levels at the G_1-S transition (39). Similar observations have been made for human BRCA1 and BRCA2 at both the mRNA and protein levels (39–46). These findings clearly demonstrate that proliferative stimuli modulate the expression of these genes. Despite the strong correlation between *Brca1* and *Brca2* expression and proliferative status, the expression of these genes also appears to be influenced by factors other than proliferation. For example, we have shown that *Brca1* and *Brca2* mRNA levels are coordinately up-regulated in postconfluent HC11 mammary epithelial cells during differentiation as well as following treatment with insulin and glucocorticoids (39). *Brca1* and *Brca2* expression increase in this setting to levels as high as those found in actively proliferating cells, despite the fact that cellular proliferation rates remain low under these experimental conditions. Together, these observations imply that *Brca1* and *Brca2* may be involved in the processes of proliferation and differentiation in the breast.

A particularly intriguing finding of our studies has been the striking degree to which *Brca1* and *Brca2* are temporally and spatially coexpressed at the mRNA level (36). We have found that *Brca1* and *Brca2* are expressed at similar levels in a similar set of tissues and in similar cellular compartments within those tissues. In fact, the developmental expression patterns of these two putative tumor suppressor genes are essentially identical during embryogenesis and in multiple tissues of the adult. This similarity is particularly evident during postnatal mammary gland development as *Brca1* and *Brca2* expression are each up-regulated during puberty and pregnancy. The coordinate induction of these genes in proliferating and differentiating mammary epithelial cells *in vitro* may provide a cellular basis for this similarity (39). These findings suggest that similar pathways and stimuli regulate the expression of *Brca1* and *Brca2* in multiple cell types. Taken together with the fact that inherited mutations in either *BRCA1* or *BRCA2* predispose mammary epithelial cells to transformation, the striking similarities in *Brca1* and *Brca2* expression patterns formed the initial basis for speculation that these genes may function in overlapping pathways and may even directly interact.

As alluded to above, no somatic mutations have been identified in *BRCA1* or *BRCA2* in sporadic breast cancers. This puzzling observation could be explained if the function of these cancer susceptibility genes in the mammary gland were restricted to specific developmental stages, as might be suggested by the tightly regulated expression that these molecules exhibit during mammary gland development. Similarly, in light of the proposed relationship between normal mammary gland development and reproductive risk factors for breast cancer, it is interesting to note that *Brca1* and *Brca2* are each up-regulated in the breast during puberty and pregnancy because these stages of development are each associated with increases in cellular proliferation as well as increases in breast cancer risk. Potentially, the induction of *Brca1* and *Brca2* expression during these developmental stages may be a protective response to proliferation or to DNA damage that accompanies proliferation, as suggested by the observation that *Rad51* is also up-regulated in proliferating cells (35, 47).

Our laboratory has chosen to focus on understanding BRCA1 and BRCA2 function in mammary epithelial cells because considerably

less is known about their function in this context and because breast cancer is the most important clinical phenotype associated with germ-line mutations in these genes. Specifically, we are interested in those aspects of mammary gland biology responsible for the observation that women carrying germ-line mutations in *BRCA1* and *BRCA2* preferentially develop cancer of the breast. Because this may ultimately relate to mammary-specific functions of these molecules, a complete understanding of the role played by these genes in breast cancer susceptibility will almost certainly require that their functions be studied directly in the mammary epithelium. As such, we are analyzing the impact of altering *BRCA1* and *BRCA2* expression levels on proliferation, differentiation, and DNA repair in the mammary epithelium using *in vivo* and *in vitro* model systems. These studies may provide insight into mechanisms of growth control and DNA damage response in normal mammary epithelial cells as well as serve as a foundation for understanding how the absence or mutation of these molecules promotes carcinogenesis.

Novel Protein Kinases

A second approach to investigating the relationship between development and carcinogenesis in the breast is to study members of a family of regulatory proteins that are typically involved in differentiation, development, and carcinogenesis. Analysis of these processes in a variety of model systems has underscored the key role frequently played by protein kinases. Many protein kinases function as intermediates in mitogenic signal transduction pathways or encode growth factor receptors whose overexpression, aberrant expression, or mutation to ligand-independent activated forms results in transformation. Several members of the protein kinase family have been shown to be involved in the development of breast cancer both in humans and in rodent model systems including the epidermal growth factor receptor, the insulin-like growth factor-I receptor, the fibroblast growth factor receptor family, HER2/Neu, Met, and Src. For instance, amplification and overexpression of *HER2/Neu* and *EGFR* have each been correlated with aggressive tumor phenotype and poor clinical prognosis. Similarly, overexpression of certain protein kinases or of their ligands in transgenic animals results in malignant transformation of the mammary epithelium. To date, however, evidence for a causal role of protein kinases in the initiation and progression of breast cancer exists for only a few members of this family of proteins. For this reason, we embarked on a screen designed to identify tyrosine kinases and serine-threonine kinases expressed in the murine breast during normal development and in breast cancer.

First-strand cDNA was prepared from mRNA isolated either from mammary glands of mice at specific developmental stages or from a series of mammary epithelial cell lines derived from breast tumors that arose in transgenic mice expressing either the activated *neu*, c-*myc*, H-*ras*, or *int2* oncogenes (48–50). Degenerate PCR was used to amplify kinase catalytic subdomains VI–IX, and the resulting cDNA clones were screened to identify those harboring catalytic domain fragments of protein kinases (51–53). This screen identified 41 kinases: 33 tyrosine kinases and 8 serine-threonine kinases, 3 of which are novel.[4] We have characterized the temporal and spatial expression of these kinases during mammary gland development as well as in a panel of mammary epithelial cell lines derived from breast tumors arising in transgenic mice expressing either the activated *neu*, c-*myc*, H-*ras*, or *int2* oncogenes.[4] This analysis has revealed that many of these kinases are preferentially expressed in the breast during specific stages of puberty, pregnancy, lactation, and postlactational regression.

Our laboratory has subsequently focused on the function of three novel serine-threonine kinases identified in our screen: *Hunk*, *Punc*, and *Krct*. The novel protein kinase, *Hunk*, was initially isolated from a mammary epithelial cell line derived from a breast tumor that arose in a transgenic mouse expressing the *neu* oncogene (54).[4,5] Analysis of sequence homology within a portion of the catalytic domain of *Hunk* suggests that it is a serine/threonine kinase with highest homology to the *SNF1* kinase family. The novel protein kinase, *Punc*, was initially isolated from the mammary glands of mice undergoing early postlactational regression.[4,6] The catalytic domain of *Punc* is 60% identical at the amino acid level to calcium/calmodulin-dependent protein kinase I and shares a lower homology with other members of the calcium/calmodulin-dependent kinase family (55).[6] *Krct* appears to represent a new family of mammalian protein kinases and is most closely related to a protein kinase recently identified by the yeast genome project that does not fall into any of the families of protein kinases previously identified in yeast (54).

Hunk and *Punc* appear to be particularly relevant to studies of the relationship between mammary gland development and carcinogenesis by virtue of their patterns of expression.[7,8] Specifically, *Hunk* is expressed at low levels in the mammary glands of immature and mature virgin animals and undergoes a dramatic up-regulation of expression during early pregnancy. *Hunk* expression rapidly drops to basal levels by midpregnancy and decreases further during lactation and early postlactational regression. Like *Hunk*, *Punc* expression is also up-regulated in the mammary epithelium during pregnancy. However, unlike *Hunk*, maximum levels of *Punc* expression occur late in pregnancy just prior to parturition.

To determine whether the developmental changes in *Hunk* and *Punc* expression observed during pregnancy represent global changes in expression occurring throughout the mammary gland or changes in the abundance of an expressing subpopulation of cells, we have defined the spatial pattern of expression of these kinases.[7,8] This was of particular interest because the expression of several protein kinases has been shown to be cell lineage restricted, thereby permitting their use as markers for biologically interesting subpopulations of cells. Examination of the spatial pattern of *Hunk* and *Punc* expression revealed that throughout the course of mammary development both kinases are expressed predominantly in the mammary epithelium. Interestingly, the expression of each of these kinases in the mammary epithelium is strikingly heterogeneous, with the greatest number of *Hunk*-expressing cells being observed at day 7 of pregnancy and the greatest number of *Punc*-expressing cells being observed at day 20 of pregnancy. This pattern of expression does not appear to be due to the heterogeneous distribution of cells through the cell cycle. Analogously, studies of the expression of these kinases in a variety of other tissues suggest that *Hunk* and *Punc* expression may also identify subsets of cells in other organs besides the breast. These observations suggest that *Hunk* and *Punc* are differentially expressed in distinct

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epithelial cell subtypes in the breast that are differentially regulated during pregnancy.

To further investigate this hypothesis, we have examined *Hunk* and *Punc* expression in a panel of mammary epithelial cell lines derived from independent mammary adenocarcinomas arising in transgenic mice expressing the *neu,* c-*myc*, H-*ras*, or *int2* oncogenes.[7,8] Surprisingly, all eight cell lines derived from breast tumors that arose in transgenic mice expressing the *neu* or H-*ras* oncogenes were found to express high levels of *Hunk* mRNA, whereas none of the seven cell lines derived from breast tumors that arose in transgenic mice expressing the *c-myc* or *int-2* oncogenes expressed detectable levels of *Hunk*. Conversely, all seven cell lines derived from breast tumors that arose in transgenic mice expressing the *c-myc* or *int-2* oncogenes expressed *Punc* mRNA, whereas none of the eight cell lines derived from breast tumors that arose in transgenic mice expressing the *neu* or H-*ras* oncogenes expressed detectable levels of *Punc*. In each case, kinase expression levels observed in tumor cells were significantly higher than those observed in nontransformed mammary epithelial cells.

The heterogeneous spatial patterns of *Hunk* and *Punc* expression in the breast, along with the mutually exclusive patterns of expression of these two kinases in transgenic mammary epithelial cell lines, suggest that these novel serine-threonine kinases may be differentially expressed in distinct mammary epithelial cell subtypes that are themselves differentially regulated during pregnancy. The observation that *Hunk* and *Punc* are overexpressed in cell lines derived from breast cancers induced by the *neu* or c-*myc* oncogenes, respectively, suggests either that *Hunk* and *Punc* are downstream targets of the *neu* and c-*myc* oncogenes or that these kinases identify epithelial cell subtypes that are preferentially transformed either by *neu* or c-*myc*.

Each of these hypotheses is based on our observations suggesting that the normal mammary epithelium appears to be composed of distinct *Hunk*- and *Punc*-expressing cell types. The first hypothesis postulates that *Hunk* mRNA expression is activated by the *neu* and/or H-*ras* pathways, whereas *Punc* mRNA expression is activated by the c-*myc* and/or *int2* pathways. In this model, *neu* (or c-*myc*) transgene expression in the mammary epithelium induces *Hunk* (or *Punc*) expression in all mammary epithelial cell types that express the transgene. As a consequence, tumors that arise from the epithelium display the same differential pattern of expression exhibited by the parental normal transgenic mammary epithelium. The second hypothesis postulates that *neu* and c-*myc* preferentially transform two different mammary epithelial cell types, one of which (in the case of *neu*) is marked by *Hunk* expression and the other of which (in the case of c-*myc*) is marked by *Punc* expression. In this model, overexpression of *Hunk* in *neu*-induced tumors reflects the selection and outgrowth of an *Hunk*-expressing epithelial cell subtype that otherwise represents a minor fraction of cells in the normal mammary epithelium. That is, *Hunk* and *Punc* expression may be restricted to distinct epithelial cell subtypes that are preferentially transformed by these oncogenes.

Our data suggest that the novel serine/threonine kinases identified in our laboratory may serve as markers for biologically interesting subpopulations of epithelial cells in the breast that are relevant both to development and carcinogenesis. Current work in our laboratory on *Hunk*, *Punc*, and *Krct* focuses on placing these kinases in known or novel signal transduction pathways and on determining their role in mammary development and carcinogenesis using transgenic and knockout animal models as well as tissue culture model systems. In addition, we have cloned the human homologues for each of these genes and are currently determining whether *Hunk*, *Punc*, and *Krct* are mutated, amplified, or overexpressed in human tumors or tumor cell lines.

Parity-induced Changes in the Breast

A third approach that our laboratory is taking to explore the relationship between development and carcinogenesis in the breast is to focus on the molecular and cellular changes that occur in the breast as a result of reproductive events known to influence breast cancer risk. Epidemiological studies have consistently shown that women who undergo an early first full-term pregnancy have a significantly reduced lifetime risk of breast cancer (1, 56–64). This association is independent of parity (*i.e.*, number of live births). In contrast, women who undergo their first full-term pregnancy after the age of 30–35 years appear to have a risk of breast cancer that is actually higher than that of nulliparous women. This suggests that parity-induced protection against breast cancer is principally dependent upon the timing of a first full-term pregnancy rather than on its occurrence *per se*. These observations imply that an early first full-term pregnancy results in a change in the breast, either directly or indirectly, that confers a decreased risk for the subsequent development of breast cancer. Because aborted pregnancies are not associated with a decreased risk for breast cancer, it has been hypothesized that the protective effect of parity requires attaining the terminally differentiated state of lactation (2, 3, 6, 59, 65–71). Unfortunately, the biological basis of parity-induced protection against breast cancer is unknown. In principle, the protective effect of early first childbirth could result from the pregnancy-driven terminal differentiation of a subpopulation of target cells at increased risk for carcinogenesis, from the preferential loss of a subpopulation of target cells during postlactational regression or from a permanent systemic endocrine change affecting the breast in such a way as to reduce the risk of carcinogenesis. Clearly, a more thorough elucidation of the molecular and cellular changes that take place in the breast as a result of parity will be required to fully understand this phenomenon.

The realization that specific reproductive endocrine events alter breast cancer risk in a predictable fashion raises the possibility that events known to decrease breast cancer risk might be mimicked pharmacologically. The desire to pursue this objective is heightened by the fact that, although it is now possible by genetic means to identify women who are at elevated risk for developing breast cancer, interventions between the extremes of more frequent mammographic screening and prophylactic bilateral mastectomy are only now beginning to be considered. As such, reducing breast cancer risk via hormonal manipulations designed to mimic naturally occurring endocrine events could represent a feasible alternative. It is to this end that both early first full-term pregnancy and early menopause have been proposed as logical paradigms on which to model the hormonal chemoprevention of breast cancer. The achievement of this goal, however, has been hampered by current ignorance regarding the mechanism by which reproductive history alters breast cancer risk. As such, the rational design of hormonal chemoprevention regimens would benefit from a better understanding of the influence of development on breast cancer risk. An additional stumbling block in the development of chemoprevention regimens aimed at reducing breast cancer risk has been the prolonged and costly clinical trials required to determine the efficacy of these regimens due to reliance on the development of breast cancer as a clinical end point (72–75). As such, the identification and use of intermediate molecular end points that accurately identify changes in the breast associated with changes in breast cancer risk would facilitate the development of such chemopreventive regimens. To this end, we have chosen to exploit the relationship between development and carcinogenesis in the breast to generate rational and biologically plausible candidate surrogate end point biomarkers.

The mechanism of parity-induced protection against breast cancer

is likely to involve complex genetic and epigenetic processes that may be influenced by reproductive endocrine variables as well as by inherited genotypes. In this context, it is useful to analyze complex processes such as this in model systems that recapitulate relevant epidemiological findings, permit critical aspects of reproductive history to be rigorously controlled, reduce genetic variation, and permit the examination of molecular and cellular events at defined developmental stages of interest in normal tissue. The use of animal models to study the impact of mammary gland development on breast cancer risk is facilitated by the fact that the structure, function, and developmental stages through which the mammary gland passes are similar in humans and in rodents (76, 77). Administration of the carcinogen DMBA to nulliparous Sprague Dawley rats induces mammary adenocarcinomas that are hormone dependent and histologically similar to human breast tumors. In contrast, rats that have previously undergone a full-term pregnancy are highly resistant to the induction of breast cancer by carcinogen administration, as compared with age-matched nulliparous controls (2, 6, 78–83).

Paralleling these functional differences, there are also marked morphological differences between the adult nulliparous mammary gland and the mammary glands of age-matched parous littermates that have undergone a single cycle of pregnancy, lactation, and regression. These parity-induced morphological changes are permanent because nulliparous and parous glands may be distinguished easily even after 1 year of postlactational regression (3).[9] Similar morphological changes are also seen in mice and in rats and are analogous to those reported in the parous human breast (70, 77). These observations support the hypothesis that parity results not only in a permanent change in the functional state of the breast (*i.e.*, susceptibility to carcinogenesis) but also in permanent structural changes in the breast. Finally, the fact that the Sprague Dawley DMBA model system mirrors complex epidemiological phenomena observed in humans, and that numerous molecules believed to play important roles in the pathogenesis of human breast cancer have similar effects in rodents, suggests that rodent model systems such as this can be a valuable tool for understanding fundamental aspects of mammary gland biology and breast cancer etiology.

We hypothesize that understanding the impact of parity on breast cancer risk will require a thorough understanding of the manner in which reproductive history affects subpopulations of cell types present in the breast. To address this hypothesis, we are using rodent model systems to identify and evaluate genes that are differentially expressed in the breast as a function of parity. Candidate genes that are specifically expressed in either the parous or the nulliparous rodent breast are being isolated and identified using a variety of approaches. These differentially expressed genes are being used as biomarkers for the cellular and molecular changes that occur in the breast as a result of an early first full-term pregnancy to define the impact of early parity on the development and differentiation of specific cell types in the breast. Finally, biomarkers that are found to be biologically informative in the rodent model system are being tested for their ability to detect parity-associated changes in histologically normal breast tissue obtained from nulliparous and parous women with known reproductive history and hormone exposures. The level and spatial pattern of expression of each of these candidate biomarkers is being analyzed in human tissue and evaluated with respect to parity as well as other parameters of reproductive endocrine history, such as age, age at first full-term pregnancy, menopausal status, and exogenous hormone use. These studies will determine whether candidate biomarkers characterized in rodent model systems can specifically detect parity-induced changes in the human breast.

To date, this approach has yielded a variety of genes that are expressed at higher levels in the mammary glands of parous animals as compared with age-matched virgin controls, confirming the utility of this approach for isolating genes that are specifically expressed in the breast as a function of reproductive history.[9] Several of the parity-specific genes that we have initially isolated are markers of mammary epithelial cell differentiation, such as milk proteins. This finding suggests that the parous breast is more "differentiated" than the nulliparous breast and, as such, is consistent with the proposal made by Russo and Russo (2, 84) that parity protects against breast cancer by virtue of the differentiation that it induces. The developmental patterns of expression of milk protein genes are notably heterogeneous because each is up-regulated at a specific point in the alveolar differentiation pathway (85). Interestingly, we have found that the expression patterns of several of these genes reflect subtle aspects of reproductive history.[9] As such, studying the regulated expression of this class of genes as a function of reproductive history may provide insights into parity-related events in the breast. In addition, we have isolated a number of genes that are as yet unidentified. Given their interesting developmental patterns of regulation and parity-specific pattern of expression, these genes appear to represent an informative pool of candidate biomarkers for detecting changes in the breast associated with reproductive events.

In theory, the parity-specific pattern of expression for a given biomarker could reflect a global increase in expression of the gene in all mammary epithelial cells, an increase in the percentage of expressing cells in the breast, or both. We are analyzing the developmental pattern of expression of candidate genes by *in situ* hybridization to distinguish between these mechanisms. Our results indicate that parity-specific patterns of expression for different genes result from distinct developmental pathways. For example, these studies reveal examples of parity-dependent global changes in expression as well as parity-dependent changes in the abundance of expressing cells. This latter example is suggestive of a permanent pregnancy-induced expansion in the number of cells expressing a given biomarker in the breast.[9] These findings are consistent with the hypothesis that reproductive events may permanently alter the biology of the breast by differentially affecting subpopulations of cells.

We have also determined the impact of several reproductive parameters on the differential pattern of expression of these genes.[9] These experiments reveal that the parity-specific pattern of expression for some genes is independent of age, duration of postlactational regression, and age at first full-term pregnancy. In contrast, other genes we have identified are expressed in a parity-specific manner in the mammary glands of animals that have been mated as adolescents but not in the mammary glands of animals that have been mated as adults. These results suggest that the regulation of expression of such genes reflects developmental events in the mammary gland that are specific for age at first full-term pregnancy. These findings suggest that candidate cDNA biomarkers generated by these approaches may provide insight into subtle aspects of the molecular and cellular changes that occur in the breast as a result of parity. Ultimately, these studies are intended to gain sufficient understanding of the molecular pathways responsible for parity-induced protection against breast cancer in order to permit this naturally occurring protective event to be mimicked pharmacologically.

Summary

The current aims of this laboratory are designed to develop the molecular tools required to understand the relationship between nor-

[9] C. M. D'Cruz, J. Wang, S. I. Ha, and L. A. Chodosh, Reproductive history results in a permanent change in the expression of specific genes in the murine breast, manuscript in preparation.

mal mammary gland development and mammary carcinogenesis, as reflected in the epidemiology of reproductive endocrine risk factors for breast cancer. We have taken three approaches toward understanding this relationship, including: determining the role normally played by breast cancer susceptibility genes in mammary epithelial development; studying the function of three novel protein kinases in the breast; and identifying and analyzing genes that are specifically expressed in the breast during developmental stages associated with changes in breast cancer risk. We anticipate that these approaches will ultimately lead to a clearer understanding of the mechanisms by which breast cancer susceptibility is modulated by reproductive history.

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Discussion

Dr. Andrew Feinberg: I have a really simple-minded question. These are very elegant studies, but I worry a bit about transgene-induced tumors, because in a sense you're starting with loaded dice. Aren't there any models of spontaneous mammary tumorigenesis? I thought there were some dogs or other species that developed cancer in a similar epidemiological manner as you mentioned for humans at the beginning. But, I don't know this field, so I may be totally wrong.

Dr. Chodosh: It is true that there are certain breeds of dogs that do develop breast cancer spontaneously, though I am not aware of any that show parity-induced protection against breast cancer. Obviously, how you choose a model to study a particular question is a central issue. Regarding transgenic rodent models of breast cancer there are a couple of points worth making.

The first is that our main experimental thrust is to look at the normal developmental biology of the breast. There is no question that the developmental stages through which the breast passes for both the mouse and the rat are exceedingly similar to what one finds in the human. That is, the developmental processes are as highly conserved as histology and tissue architecture.

The second is that it's quite clear from transgenesis experiments that many of the pathways that are altered during the process of carcinogenesis in the human breast cause similar problems in the rodent breast when altered by transgenic approaches. That is, the molecular pathways involved are highly conserved. So, while tumor development in a transgenic system is not "spontaneous" in the same way that we think of for human breast cancers, I would argue that the

history of cancer biology suggests that they are still quite useful models to examine pathways involved in development and carcinogenesis. So at the moment, as far as animals that we can work with, particularly those that we can genetically manipulate, we have mice. Similarly, in the rat, one is somewhat restricted to carcinogen-induced models, which may or may not faithfully mimic the processes involved in human carcinogenesis.

We think about the suitability of our model systems a great deal, and it's not clear to me that there's another *in vivo* system available at the present time that's more appropriate.

Speaker: Do you have any evidence these kinases play similar roles in the human breast? Because human breast cancer is quite different. Pathological studies are quite different from real breast cancer, because it's quite complicated by different pathways. So, my interest at the moment is that even if we are able to link these kinases to the set of human reactants, it is different with different types of breast cancer and different kinases being expressed. How do you plan to address these potential differences?

Dr. Chodosh: A very important question, which explains why we are moving into human tissue and human breast cancer cell lines to address some of these issues. This is information that we're currently gathering. The data that I showed you in human breast cancers and cancer cell lines are quite recent, so it's too preliminary for us to know whether there is some correlation between the expression of our kinases and Erb2 status or ER status, or a particular histological cell type. Regarding tumors that are marked by *Hunk* or *Punc* expression, clearly we would want to know whether they behave differently in terms of patient prognosis or response to therapy. We don't know that yet, though that's certainly something that we're very interested in.

Dr. Robert Ryan: I would like to ask, have you considered perhaps doing something like the chip-based assay where now you use the MMTV-neu and MMTV-c-myc breast cancer cell lines and test those samples for changes by looking at the various genes that are up-regulated or down-regulated. It might give you a handle on that, do you think?

Dr. Chodosh: Yes, that's certainly a possibility. In the context of DNA chip technology, I think we'd probably want to make the fewest possible changes that we could, starting with the most normal cells we can, then induce expression of a *Hunk* or *Punc* transgene and ask what genes are downstream, as opposed to using as a starting point tumor cell lines that obviously have undergone many unrelated changes over the long period of time they have been in culture. Certainly, I agree it's an important new technology.